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Rapid cold hardening capacity in five species of coleopteran pests of stored grain

Charles S. Burks*, David W. Hagstrum

USDA Agricultural Research Service Grain Marketing & Production Research Center, 1515 College Ave, Manhattan, KS 66502, USA

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Abstract

The capacity for rapid cold hardening was examined in recently-eclosed adults of five coleopteran species associated with stored grain. Supercooling points indicated that mortality was predominantly due to chilling injury. Threshold temperatures for indirect chilling injury for each of these species were determined by fitting survivorship data for unacclimated insects held for 0–6 h at various sub-0°C temperatures to a response surface. These response surfaces were used to predict a threshold temperature for each species at which 50% of the unacclimated individuals would survive for 2 h. The survival time at this threshold temperature with acclimation for 2 h at 4°C was compared to that without acclimation. The ratio of the 50% survivorship time of unacclimated to that of acclimated beetles was 8.7 for *C. ferrugineus*. This exceeded by over two-fold the range of 2.6–4.0 observed for the remaining species indicating that, in addition to greater innate cold hardiness, *C. ferrugineus* also has a greater capacity for rapid cold hardening than the other species examined. © 1998 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Cold tolerance of insects at sub-0°C can be increased by short exposures (e.g., 2 h) to temperatures of 0–5°C. This phenomenon has been called “rapid cold hardening” (Lee et al., 1987) to distinguish it from the seasonal acclimation or acclimatization processes in insects,

* Author for correspondence. Horticultural Crops Research Laboratory, Fresno, California 93727. Tel.: +1-209-453-3029; Fax: +1-209-453-3088; E-mail: cburks@asrr.arsusda.gov

which typically occur over periods of weeks (Lee, 1991). Rapid cold hardening was originally demonstrated in four species from the orders Coleoptera, Diptera, and Hemiptera (Lee et al., 1987), and has subsequently been demonstrated in several other insect species (e.g., Chen et al., 1990; Coulson and Bale, 1990; Czajka and Lee, 1990; Larsen et al., 1993; Rosales et al., 1993; Larsen and Lee, 1994). Interspecific comparisons have been made among dipteran species within the family Sarcophagidae (Chen et al., 1990) and of the genus *Musca* (Rosales et al., 1993), and within the homopteran genus *Dalbulus* (Larsen et al., 1993). But quantitative comparative measurements, such as the exposure time predicted to result in 50% survivorship for a given temperature (LT_{50}), were not provided for the dipteran data. Interspecific comparisons among the homopteran data demonstrated seasonal differences in cold tolerance rather than rapid cold hardening. Because questions have been raised concerning the ecological and evolutionary significance of the rapid cold hardening phenomenon (Coulson and Bale, 1990, 1991), more interspecific comparisons are warranted.

Increased tolerance of sub-0°C cold in these studies was not a result of changes in the supercooling point; thus, rapid cold hardening affected susceptibility to chilling injury rather than internal freezing. Chilling injury, i.e., cold injury in the absence of internal ice formation, can be direct or indirect (Morris and Watson, 1984; Lee, 1991). Direct chilling injury refers to acute injury and is thought to be due to damage to cell membranes (Morris and Watson, 1984), whereas indirect chilling injury refers to delayed mortality and is thought to be due to irreversible damage to cellular respiration (Morris and Watson, 1984; Hochachka and Dunn, 1986). In many single-celled organisms and single cells or cell lines from multi-cellular organisms, the temperature at which death from cold injury occurs with exposures of ≤ 2 h is a good predictor of whether injury is direct or indirect (Morris and Watson, 1984). Thus, this threshold for indirect chilling injury ($Threshold_{ICI}$) offers a way to compare cold injuries which produce a similar impact between species differing in innate (i.e., unacclimated) cold tolerance.

Insect pests of stored grain are a logical model system with which to examine the ecological significance of the rapid cold hardening process. Because many of these species are easily reared in the laboratory and the cold hardiness of these pests is economically important, there is a large amount of literature on cold hardiness in stored product insect pests (Howe, 1965; Evans, 1983; Fields, 1992). Some of these species can also be viewed as an ecological guild. For example, the species we examined—*Cryptolestes ferrugineus* (Stephens) and *Oryzaephilus surinamensis* (L.), (Cucujidae); *Rhyzopertha dominica* (F.) (Bostrichidae), *Sitophilus oryzae* (L.) (Curculionidae); and *Tribolium castaneum* (Herbst) (Tenebrionidae)—are all commonly found in wheat stored in the US Plains states. All stages may overwinter, and no overwintering diapause has been found in these species. Young adults of *R. dominica* are more tolerant of prolonged cold temperature than larvae (Hagstrum and Flinn, 1994), and the adult is the most cold-tolerant stage of *C. ferrugineus* (Smith, 1970). We wished to compare the magnitude and assess the variation of rapid cold hardening capacity in these five species that, in their usual habitat, are sheltered from both low sub-0°C temperatures and large, rapid, temperature fluctuations. In this habitat, there would seem to be little selection favoring rapid cold hardening and one would predict a uniformly low capacity for rapid cold hardening compared to species subjected to greater environmental fluctuations.

In this study, we compare the magnitude of the rapid cold hardening response in young adults of five species. To distinguish between chilling and freezing injury, we examined

supercooling points. In order to estimate the Threshold_{ICI}, we first used a previously described model (Hagstrum and Flinn, 1994) to help find the temperature for which a 2-h exposure resulted in 50% survival. The effect of rapid cold hardening (acclimation for 2 h at 4°C) was compared among these species. In order to make quantitative comparisons of the rapid cold hardening effect, we examined the ratio between the LT₅₀ values for acclimated vs. unacclimated beetles.

2. Materials and methods

2.1. Insects

The *R. dominica* colony used in this study was established from adults collected in Dickinson County, Kansas within a year of the study. Other species were from laboratory colonies which had long been maintained in the Grain Marketing and Production Research Center in Manhattan, Kansas. *Tribolium castaneum* were reared on flour with 5% (weight:weight) brewer's yeast added (*Tribolium* diet). *Oryzaephilus surinamensis* were reared on a diet of two parts rolled oats and one part *Tribolium* diet (volume:volume). *Cryptolestes ferrugineus* were reared on one part crimped to two parts uncrimped wheat with one part per thousand each of ground wheat germ and brewer's yeast. *Sitophilus oryzae* were reared on whole wheat (hard red winter, moisture content ca. 13%). *Rhyzopertha dominica* were reared on the same wheat, with ca. one part per thousand *Tribolium* diet added. *Cryptolestes ferrugineus* were reared at 32°C; the other species were reared at 27°C. All species were reared under 16:8 h L:D photoperiods. Crystallization temperatures and cold mortality were determined using newly eclosed adults for all species. Determinations on *C. ferrugineus* were approximately 30 days post-oviposition; for other species determinations were made approximately 40 days post-oviposition.

2.2. Supercooling point determinations

Supercooling points were determined using an 80286 PC, a DAS-TC analogue-to-digital board (Omega Instruments, Stamford, CT) and the DOS (Microsoft, Redmond, CT) software that came with the board. Insects, with or without rapid cold hardening, were held in contact with the end of a 30-gauge copper–constantan thermocouple. The larger species (*R. dominica*, *S. oryzae* and *T. castaneum*) were immobilized by placing them in the bottom of a 0.5 ml microcentrifuge tube capped with foam rubber, whereas the smaller species (*C. ferrugineus*, *O. surinamensis*) were immobilized by being enveloped in transparent tape. The immobilized insects were placed in glass tubes, and the tubes were then lowered into a programmable refrigerated bath containing 95% ethanol, and the temperature reduced from 4 to –40°C at a rate of 0.5°C/min. Thermocouples were monitored at 1 s intervals and the readings were saved as a text file. Exotherms (transient increases in temperature indicating crystallization of supercooled water to ice), were found by using SAS data steps to find either: (1) the lowest individual temperature recordings which exceeded the previous reading by 0.5°C; or (2) a series of ten recordings which exceeded the 10th reading prior to the first of this series by 0.2°C. These putative supercooling points were confirmed by plotting the data using SAS/GRAPH.

2.3. Cold mortality assays

The cold tolerances of these species were determined by exposing unacclimated insects for 0–6 h to temperatures of -14 to -7°C . Ten insects each were held in 1 ml of wheat in a 13×100 mm glass tube inside a 19×150 mm glass tube containing 2 ml of saturated sodium chloride (for maintenance of relative humidity near 75%), placed in a pre-chilled refrigerated bath and allowed 10 min to equilibrate, and then removed at selected intervals. Survivorship was observed 12–24 h following removal of the tubes from the cold bath. Insects were scored as dead only if no life could be detected; impaired insects were considered alive. Most treatments that resulted in mortality also left additional insects impaired; this definition of ‘alive’ was chosen because it fitted the logit model better than a more stringent one. Capacities for rapid cold hardening were determined by exposing insects to the $\text{Threshold}_{\text{ICI}}$ for various times with or without prior acclimation at 4°C for 2 h.

2.4. Statistical analysis

Data were analyzed using SAS 6.11 (SAS Institute Inc., Cary, North Carolina). A 2-way ANOVA (PROC ANOVA) with acclimation and replicate as the main effects was used to examine whether rapid cold hardening affected the crystallization temperature. The response surface equation described by Hagstrum and Flinn (1994) was used to estimate $\text{Threshold}_{\text{ICI}}$:

$$\text{Survivorship} = 1 / \left(1 + e^{-f(\text{ET})} \right)$$

where

$$f(\text{ET}) = X_0 + X_1 * \text{min} + X_2 * \text{min}^2 + X_3 * \text{min}^3 + X_4^{*^{\circ}\text{C}} + X_5^{*^{\circ}\text{C}^2} + X_6 * \text{min}^{*^{\circ}\text{C}}.$$

PROC PROBIT was used to obtain parameter estimates for X_0 – X_6 . Table Curve 3D (Jandel Scientific, San Rafael, CA) was used to obtain predictions and confidence intervals. In order to compare the LT_{50} , PROC PROBIT was used to calculate logistic regressions for unacclimated and acclimated animals.

3. Results

Median crystallization temperatures varied between species (Fig. 1), but 2-way ANOVA revealed no statistically significant effects of acclimation on supercooling points. The highest supercooling points were found among *R. dominica*, in which 19% of the individuals tested (12/58) froze internally at temperatures higher than -12°C . Among the other species examined, the 90th percentile supercooling point was less than the temperature at which cold survivorship was examined ($\geq -14^{\circ}$ for *C. ferrugineus*, $\geq -12^{\circ}\text{C}$ for the other species).

The equation used to fit a response surface provided a reasonably good fit for *O. surinamensis*, *R. dominica*, *S. oryzae*, and *T. castaneum*, with r^2 values ranging from 0.77 to 0.84 (Table 1). The fit was poorer for *C. ferrugineus*, with a r^2 value of 0.23. Comparison of

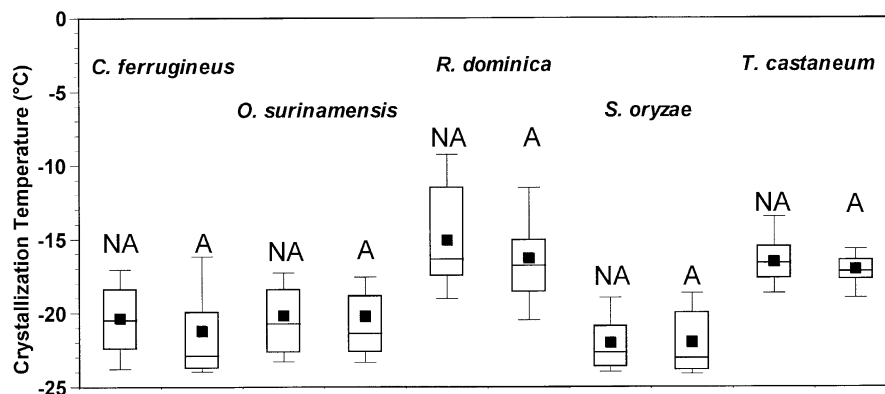


Fig. 1. Distribution of crystallization temperatures for five coleopteran pests of stored grain products. The center bars represent the median, the squares represent the mean, the upper and lower box ends mark the 25th and 75th percentile, and the whisker marks the 10th and 90th percentile. The signs represent unacclimated (NA) and acclimated (A) insects. Each box represents 26–30 observations. There is no significant acclimation effect (2-way ANOVA, data not shown).

the response surface for *C. ferrugineus* (Fig. 3) with those of the other species (Figs. 2 and 3) reveals a more rapid decrease in survivorship over the lowest 1°C range (i.e., -13 to -14°C), as well as greater overall variability. Generally a plateau in the response surface representing 100% survivorship of individuals exposed to cold seemed to occur earlier for the internal feeders, *S. oryzae* and *R. dominica* (Fig. 2) than for the external feeders, *T. castaneum*, *O. surinamensis*, and *C. ferrugineus* (Fig. 3).

The Threshold_{ICI} estimates obtained from the response surface were used as starting values to find a threshold temperature at which a 2 h exposure reliably produced ca. 50% survivorship in unacclimated insects. Rapid cold hardening capacities of these species were compared using logit analysis to determine an LT₅₀ with and without prior acclimation at 4°C. The largest ratio of acclimated to unacclimated LT₅₀ occurred in *C. ferrugineus* (Table 2).

4. Discussion

This study differs from previous examinations of rapid cold hardening in using the temperatures with the same physiological impact for each species to compare their capacity for rapid cold hardening, rather than the same temperature. In the current and previous studies (Howe, 1965; Fields, 1992), *C. ferrugineus* was more cold hardy than the other species. Our findings indicate that *C. ferrugineus* also has a greater capacity for rapid cold hardening in young adults than other species.

Rapid cold hardening was due entirely to increased resistance to chilling injury; it did not affect supercooling points. In four of the species studied, 90% of individuals examined supercooled to temperatures below those at which non-freezing cold mortality (i.e., chilling injury) occurs within 2 h and there was no difference between the crystallization temperatures of rapid cold-hardened and unacclimated individuals. In *R. dominica*, 19% of the individuals examined froze internally within the range of -12 to -8°C in which cold mortality occurred.

Table 1
Response surface parameters

Species	Parameter										r^2	n
	X_0	X_1	X_2	X_3	X_4	X_5	X_6					
<i>C. ferrugineus</i>	5.308 ± 6.864	-0.0419 ± 0.017	$0.000164 \pm 7.15 \times 10^{-3}$	$-2.74 \times 10^{-7} \pm 1.32 \times 10^{-7}$	0.255 ± 1.083	-0.00237 ± 0.0429	-0.000810 ± 0.000866				0.23	78
<i>O. surthamensis</i>	-65.0 ± 30.5	0.0422 ± 0.0477	$0.000170 \pm 9.39 \times 10^{-5}$	$-2.701 \times 10^{-7} \pm 1.53 \times 10^{-7}$	-14.2 ± 5.78	-0.729 ± 0.271	0.00910 ± 0.0397				0.79	107
<i>R. dominica</i>	-20.3 ± 8.16	0.0831 ± 0.0173	$-3.31 \times 10^{-5} \pm 4.79 \times 10^{-5}$	$-3.20 \times 10^{-9} \pm 5.82 \times 10^{-8}$	-4.52 ± 1.57	-0.221 ± 0.0741	0.00943 ± 0.00141				0.82	107
<i>S. oryzae</i>	-20.4 ± 6.20	0.0593 ± 0.0144	$5.41 \times 10^{-5} \pm 4.79 \times 10^{-5}$	$-1.04 \times 10^{-7} \pm 9.10 \times 10^{-8}$	-4.82 ± 1.42	-0.249 ± 0.0795	0.0103 ± 0.00152				0.84	84
<i>T. castaneum</i>	-19.4 ± 21.2	0.182 ± 0.0521	-0.000190 ± 0.000114	$-2.56 \times 10^{-7} \pm 1.92 \times 10^{-7}$	-4.6 ± 4.26	-0.241 ± 0.211	-0.0193 ± 0.00452				0.77	74

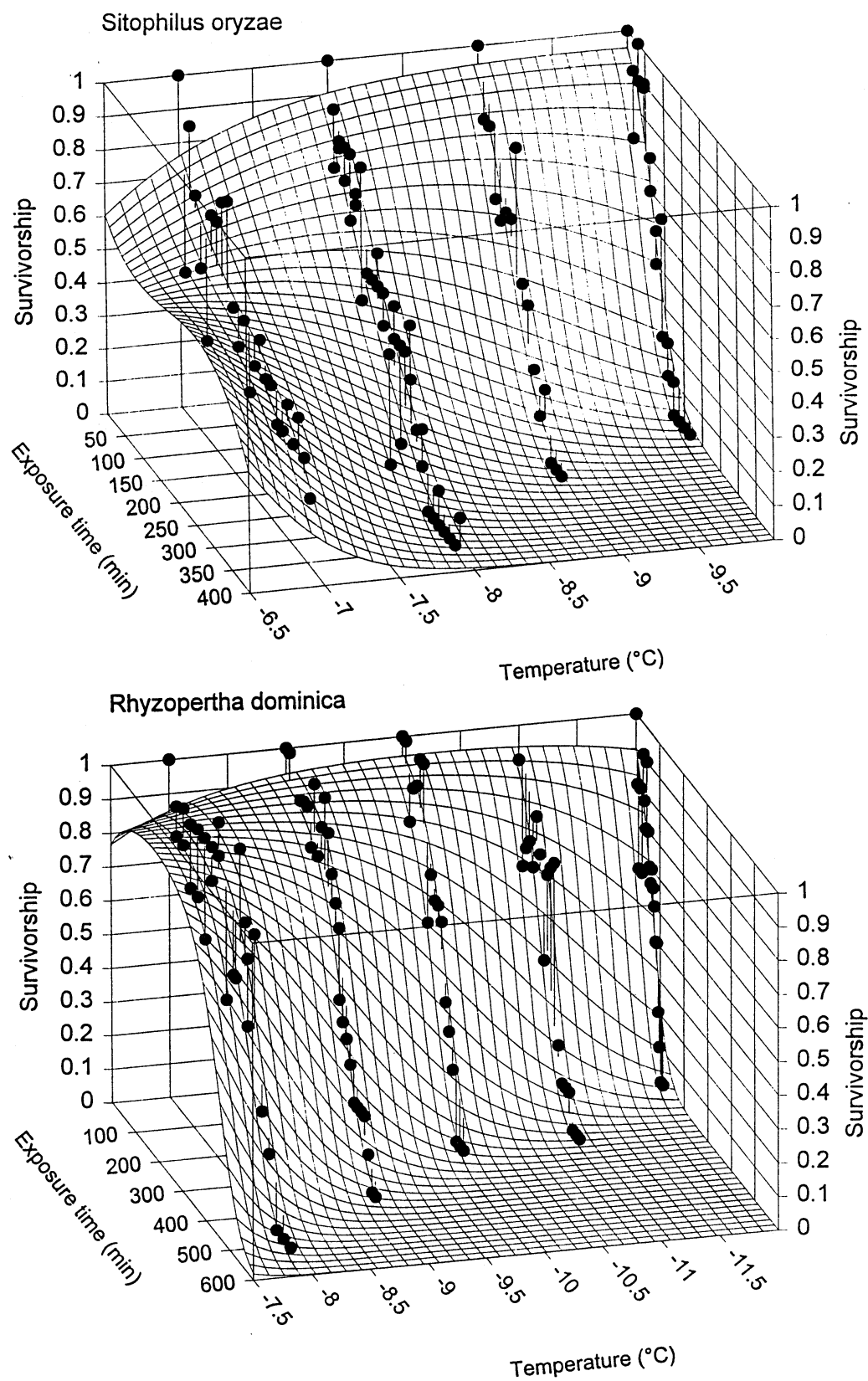


Fig. 2. Observations (●), and fitted response surfaces (—) for *S. oryzae* and *R. dominica* as a function of °C and time. Each point represents 10–20 insects.

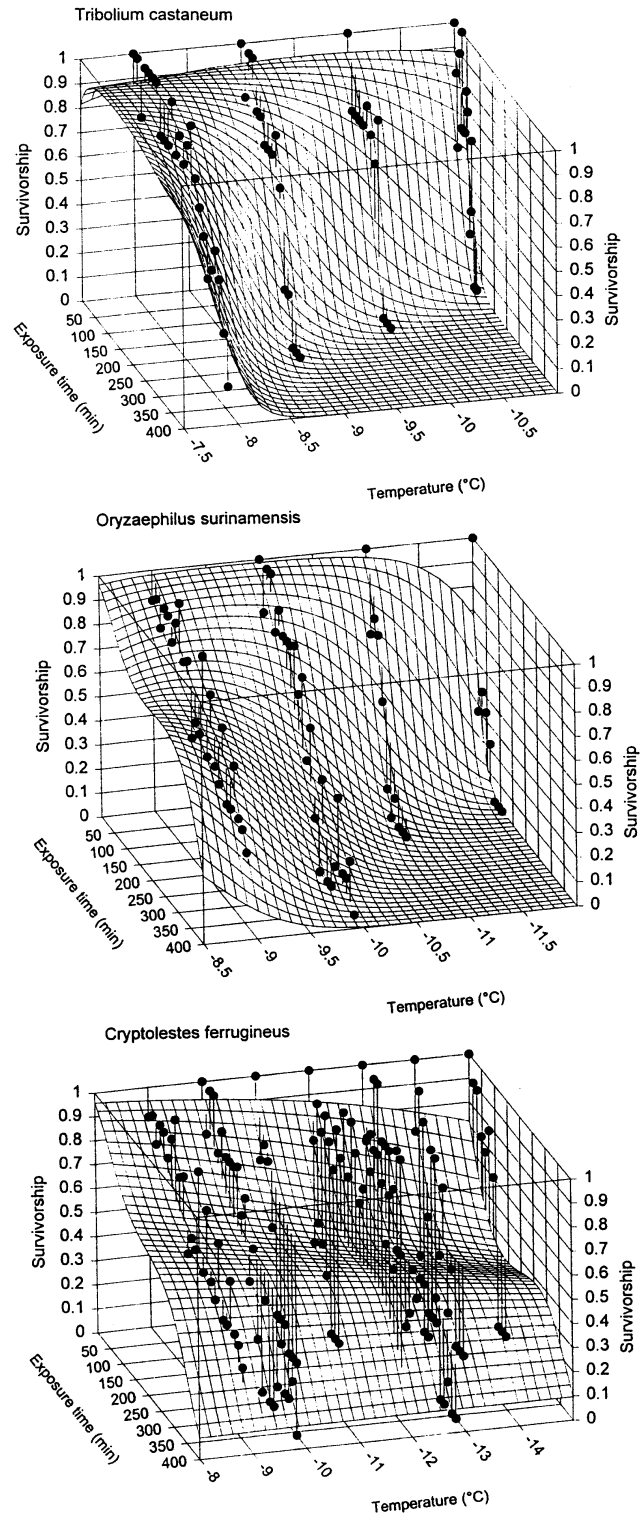


Fig. 3. Observations (●), and fitted response surfaces (—) for *T. castaneum*, *O. surinamensis*, and *C. ferrugineus* as a function of °C and time. Each point represents 10–20 insects.

Table 2

Exposure temperatures for the five species, predicted survivorship, 95% confidence intervals, and ratio of unacclimated chill tolerance

Species	Temperature (°C)	Treatment	n	slope \pm SE	χ^2	LT ₅₀ (min) (95% CI)	Chill Tolerance Ratio
<i>Sitophilus oryzae</i>	−7.5	Unacclimated	26	−0.014 \pm 0.0018	21.31	104 (79,125)	3.0
		Acclimated	21	−0.0069 \pm 0.00025	13.39	313 (274,352)	
<i>Oryzaephilus surinamensis</i>	−9.6	Unacclimated	25	−0.0097 \pm 0.0014	17.74	194 (163,225)	2.3
		Acclimated	25	−0.0080 \pm 0.0014	28.24	500 (392,870)	
<i>Tribolium castaneum</i>	−9.8	Unacclimated	16	−0.025 \pm 0.0035	17.15	149 (136,162)	4.0
		Acclimated	29	−0.0043 \pm 0.00051	36.69	589 (553,627)	
<i>Rhyzopertha dominica</i>	−10.7	Unacclimated	12	−0.014 \pm 0.0018	21.31	59 (37,77)	3.7
		Acclimated	25	−0.013 \pm 0.00097	25.37	217 (191,248)	
<i>Cryptolestes ferrugineus</i>	−14.0	Unacclimated	19	−0.019 \pm 0.0035	30.29	157 (128,191)	8.7
		Acclimated	15	−0.0017 \pm 0.00025	12.97	1370 (1121,1649)	

However, more acclimated than unacclimated beetles had high crystallization temperatures. This indicates that in *R. dominica*, as in other species in this and previous studies, depression of the crystallization temperature was not involved in the increased survivorship resulting from rapid cold hardening.

The increase in survival time by a factor of 2.3- to 3.7-fold in *O. surinamensis*, *R. dominica*, *S. oryzae* and *T. castaneum* is in range with rapid cold hardening capacities implied by other comparative studies. Examination of the data from the studies comparing dipteran rapid cold hardiness suggests LT₅₀ ratios of ca. 2.5-fold for *Musca domestica* (L.) and 4-fold for nondiapauses or diapause-induced *M. autumnalis* (De Geer) (Rosales et al., 1993). The data for the sarcophagid species suggest at least a 4-fold increase in LT₅₀ in rapid-cold hardened individuals for the temperate and high-altitude species *Sarcophaga bullata* (Parker), *S. crassipalpis* Macquart, and *Blaesoxipha plinthopyga* (Wiedeman), and little or no increase in LT₅₀ as a result of rapid cold-hardening in the low elevation tropical species *Peckia abnormis* (Enderlein) and *Sarcodexia sternodontis* Townsend (Chen et al., 1990). The sarcophagid species overwinter as pupae in the upper soil layers and would probably be somewhat protected from fluctuations in air temperature (e.g., soil temperatures reported in Burks et al., 1996). The muscid species overwinter as diapausing adults or as continuously reproducing populations and would probably, therefore, be exposed to greater temperature fluctuations than sarcophagids. Therefore, a comparison of the current findings with previous reports does not suggest correlation between rapid cold hardening capacity and the degree to which a species is exposed to fluctuating low temperatures in its overwintering hibernaculum.

Within stored products insects, comparisons between the current study and a previous study of the same species and stage suggest differences between rapid cold hardening and more gradual cold acclimation, and between cold hardiness at sub-0 and above-0°C. The previous study examined cold tolerance of those species at 9°C without acclimation, or with gradual acclimation over a period of three weeks (Evans, 1983). Subsequent analysis of those data suggests a ranking of cold tolerance among those species of *O. surinamensis* > *C. ferrugineus* > *S. oryzae* > *R. dominica* > *T. castaneum* (Hagstrum et al., 1995). Based on our estimate of the Threshold_{ICI}, the order of cold hardiness is *C. ferrugineus* > *R. dominica* > *T. castaneum* = *O. surinamensis* > *S. oryzae*. In that previous study, the ratio of the LT₅₀s of acclimated-to-unacclimated insects at 9°C was *C. ferrugineus*, 10.7; *T. castaneum*, 8.2; *S.*

oryzae, 6.9; *O. surinamensis*, 5.2; and *R. dominica*, 4.4. Comparison of these ratios with our chill tolerance ratio indicates that *C. ferrugineus* and *R. dominica* do not receive as much additional benefit as the other species from gradual acclimation compared to rapid cold hardening.

Response surfaces were used as a means of estimating the threshold for indirect chilling injury in these species. The equation used to generate these response surfaces was the same as one used earlier to examine survivorship of overwintering *R. dominica* at higher temperatures and over longer periods of time (Hagstrum and Flinn, 1994). The relatively high r^2 values of the response surfaces for *O. surinamensis*, *R. dominica*, *S. oryzae*, and *T. castaneum* indicate that this equation provides a reasonable comparison between these species. While in some cases the surface ‘turns down’ at the upper edge of the range examined (e.g., *S. oryzae* and *R. dominica*), the surfaces for these species otherwise predict fairly reliably within the range of the data used to fit them.

The greater innate tolerance of sub-0°C temperatures and capacity for cold-hardening in *C. ferrugineus* compared to the other species examined suggests that, at least among these species, similar factors are involved in both resistance to chilling injury at sub-0°C temperatures and rapid cold hardening. Further studies to elucidate these factors are warranted.

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